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# HYDRAZINE AND THE METHYLATION OF DNA GUANINE

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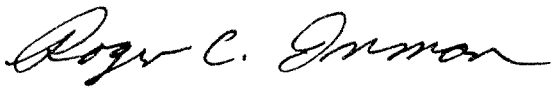

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Hydrazine, a strong reducing agent widely used in industry and the military, is hepatotoxic and weakly carcinogenic. Earlier studies demonstrated that hydrazine administration to rodents results in the methylation of liver DNA guanine and that S-adenosylmethionine was the apparent methyl donor in this response. The present study characterized the hamster as an animal model in a study of the methylation response to acute and subchronic exposures to hydrazine and has examined further the possible roles S-adenosylmethionine and the 1-carbon pool may have in the methylation of liver DNA guanine in hydrazine-poisoned animals.

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The hamster is more resistant than the rat or mouse to the acute toxicity of hydrazine and can tolerate up to 120 mg hydrazine/kg body wt per os. The amounts of 7-methylguanine and $O^6$-methylguanine that formed in liver DNA changed little when the dose of hydrazine (<45 mg/kg) produced little cytotoxicity; however, at higher doses (>60 mg/kg) the formation of these aberrant bases in liver DNA increased greatly, as did the cytotoxicity in the liver. The rate of formation of these bases after administration of 90 mg hydrazine/kg body wt was rapid and $O^6$-methylguanine persisted in hamster liver DNA longer than it did in rat liver DNA, suggesting that repeated doses of hydrazine to the hamster may force an accumulation of this promutagenic lesion in the DNA. Hamsters were given hydrazine sulfate in the drinking water to provide daily doses of 6, 11, and 17 mg hydrazine/kg body wt. These doses caused an 80-90% reduction in body wt, compared to controls, after 9 weeks exposure and thus approached a maximum tolerated dose. Both 7-methylguanine and $O^6$-methylguanine were detected in liver DNA throughout the 9-week treatment; moreover, $O^6$-methylguanine accumulated in liver DNA of those hamsters receiving the two higher doses, but not the lowest dose of hydrazine. This strong accumulation of $O^6$-methylguanine (the $O^6$-methylguanine:7-methylguanine ratio approached 1 by the 9th week) would suggest that chronic administration of hydrazine at the two higher doses should induce liver cancer in the hamster, even though reports from other laboratories which have tested hydrazine for carcinogenicity in the hamster have failed to detect malignancies. Other toxicants were studied to determine whether agents other than hydrazine could induce the aberrant methylation of DNA. In vivo test systems involving ethanol and brain DNA and aflatoxin $B_1$, phosphorus, and diethylnitrosamine and liver DNA failed to demonstrate formation of methylguanines. Some 7-methylguanine was detected in liver DNA of rats and hamsters given N-nitrosopyrrolidine but the response was not dose-related. Little evidence was obtained, then, to support the earlier suggestion that the aberrant methylation of DNA may be a non-specific response to cytotoxicity. The nonenzymatic methylation of naked calf thymus DNA incubated with S-adenosylmethionine was demonstrated in a collaborative study with Dr. Louis R. Barrows and Dr. Peter N. Magee of the Fels Research Institute, Temple University School of Medicine, in Philadelphia, Pennsylvania. The formation of 7-methylguanine, but not $O^6$-methylguanine, was confirmed but the extent of methylation was insufficient to explain quantitatively that which is seen in vivo in response to hydrazine administration. In addition, guinea pigs were given methionine to expand their hepatic pools of S-adenosylmethionine 20-fold, but this greater concentration of S-adenosylmethionine resulted in neither methylation of guinea pig liver DNA guanine nor an increase in the amounts of methylguanines in liver DNA in response to hydrazine administration. Previous studies were unable to obtain evidence to support the hypothesis that hydrazine in itself methylated in vivo to monomethylhydrazine which has been shown to be metabolized to an active methylating agent. The present study began a preliminary investigation to determine whether hydrazine might react with endogenous formaldehyde to form the corresponding hydrazone which in turn could be oxidized to a methylating agent, such as diazomethane. The little evidence obtained so far does not support an important role for endogenous formaldehyde in hydrazine-induced methylation of DNA guanine.

## PREFACE

This is the annual report of the subprogram on Comparative Biochemistry and Metabolism, Part 1: Carcinogenesis,and concerns work performed by the Department of Community and Environmental Medicine of the University of California, Irvine, on behalf of the Air Force under Contract Number F33615-80-C-0512, Work Unit 63020115. This document describes the accomplishments of the subprogram from June 1982 through May 1983.

R. C. Shank, Ph.D., was principal investigator for the subprogram. Acknowledgement is made to E. J. Hunt and W. F. Pool for their significant research contributions and assistance in the preparation of this report. M. Pinkerton, Toxicology Branch, was the technical monitor for the Aerospace Medical Research Laboratory.

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## INTRODUCTION

Hydrazine ($NH_2$-$NH_2$) is a strong reducing agent that is widely used in industry and by the military. The compound is acutely toxic to the liver, kidney and central nervous system (Witkin, 1956; Amenta and Johnston, 1962; Scales and Timbrell, 1982). Hydrazine is rapidly oxidized in the body to nitrogen ($N_2$) and diimide (HN=NH) and is acetylated to mono- and diacetylhydrazine ($CH_3CONHNHCOCH_3$); approximately 35% of the hydrazine is expired as nitrogen and 30-40% is excreted in the urine as unreacted hydrazine and hydrolysable conjugates (Dost et al., 1979; Nelson and Gordon, 1980).

Under appropriate conditions, hydrazine exposure can result in an increase in cancer incidence in the test animal population. One exposure condition that appears necessary for cancer induction is chronic irritation or toxicity. In a chronic inhalation study on hydrazine in three rodent species, squamous cell carcinomas of the nasal turbinates were detected only in the group with the highest exposure, which produced severe rhinitis with focal hyperplasia and squamous metaplasia early in the experiment (MacEwen et al., 1981). Upon oral administration at a dose which was growth-depressing, hydrazine induced hepatocellular carcinomas in male rats (Severi and Biancifiori, 1968) hepatocellular carcinomas (Biancifiori, 1970) and pulmonary adenocarcinomas (Severi and Biancifiori, 1968; Biancifiori and Ribacchi, 1962) and an increased incidence of lung adenomas (Yamamoto and Weisburger, 1970) in mice.

Most chemical carcinogens examined so far have proven to form highly reactive electrophiles in target tissue, resulting in covalent binding of the carcinogen to DNA and other macromolecules (Miller and Miller, 1977). DNA adducts are potentially damaging to the genetic material, and through a variety of suggested mechanisms, thought to lead to mutation in the somatic target cells. No adduct formation between hydrazine and DNA under near physiological conditions has been demonstrated, but formation of methylated guanines in DNA following hydrazine administration has been demonstrated, first by Dr. Saul Villa Trevino (see Quinter Ruiz et al., 1981) and then in our laboratory (Barrows and Shank, 1978; 1981), and such adducts are thought to be relevant to carcinogenesis (Lawley, 1976). There has been some suggestion in the several studies conducted in our laboratory so far, that the methylation of liver DNA in hydrazine-treated animals may be causally linked to the hepatotoxicity of the chemical agent (Barrows and Shank, 1981; Becker et al., 1981; Bosan and Shank, 1983). If it can be proven that DNA methylation resulting from hydrazine exposure is indeed linked to hydrazine-induced hepatotoxicity, and that DNA methylation is causally linked to carcinogenesis, then it may be proposed that exposures to hydrazine which are below those which cause toxicity would be unlikely to induce cancer.

Hydrazine is not the only compound for which an association appears between toxicity and carcinogenicity. Rats (Reuber and Glover, 1967; 1970), mice (Edwards, 1941; Edwards and Dalton, 1942; Eschenbrenner and Miller, 1945) and hamsters (Della Porta et al., 1961) develop liver tumors after receiving liver-necrotizing doses of carbon tetrachloride repeatedly for several weeks but not when the exposure is to non-necrotizing doses. Vinylidene chloride at renal-necrotizing doses produces adenocarcinomas in mouse kidney but not in rat or hamster kidney when these animals are given non-necrotizing doses (Maltoni, 1977; Maltoni et al., 1977). Rats exposed by inhalation to formaldehyde vapors at 15 ppm, 6 hours/day, 5 days/week for 18 months developed highly irritated nasal epithelial tissue including papillary hyperplasia and squamous atypia and then squamous cell carcinomas (Swenberg et al., 1980). Rats exposed to 2 or 6 ppm formaldehyde developed less severe nasal irritation and no cancer; mice exposed to the same concentrations of formaldehyde failed to produce any nasal tumors. A similar experiment with hydrazine using rats, mice, and hamsters (cited above) also produced carcinomas of the nasal turbinates only in rats at

only that dose which was severely irritating to the nasal epithelium; exposure at lower doses, which were less irritating, failed to produce the tumors (MacEwen et al., 1981). The studies with hydrazine, then, may serve as a model in biochemical investigations of the causal relationship, if any, between toxicity and carcinogenicity.

The present report summarizes further work done on the kinetics of the formation and persistence of methylated DNA guanine in liver after single and repeated administration of hydrazine to the hamster, and the importance of the hepatic pool of S-adenosylmethionine in the DNA methylation process, the ability of hepatotoxins other than hydrazine to induce this DNA methylation and the effect of the 1-carbon pool on DNA methylation in hydrazine toxicity.

## RESEARCH PROGRAM

### Methods Development

Most of the studies on DNA methylation in response to hydrazine administration have focussed on the formation and persistence of 7-methylguanine and $O^6$-methylguanine. It is desired to expand the studies to include methylation of pyrimidines as well, especially the methylpyrimidine normal to mammalian DNA, 5-methylcytosine. A method to examine DNA pyrimidines was developed for high pressure liquid chromatography and is based on release of stable pyrimidines from DNA by perchloric acid hydrolysis and by formic acid hydrolysis.

#### Perchloric Acid Hydrolysis

DNA was first partially depurinated by neutral thermal hydrolysis (5 mg DNA per ml 10 mM sodium cacodylate, pH 7.0, 100°C, 1 hr), precipitated with 0.11 volume 1 M HCl, and then completely depurinated by mild acid hydrolysis (5 mg DNA per ml 0.1 M HCl, 70°C, 30 min); this mild acid hydrolysate was taken to dryness in a rotary evaporator. Perchloric acid (68%; 30 µl/mg original DNA) was added to the dried hydrolysate and refluxed for 1 hr at 100°C. After the perchloric acid hydrolysate cooled, the perchlorate ion was precipitated with 50% KOH. The supernatant was diluted with water and acidified (pH 2) with phosphoric acid.

#### Formic Acid Hydrolysis

As above, mild acid DNA hydrolysate was taken to dryness in a sealable ampule and dissolved in 30 µl of 88% formic acid/mg original DNA. The ampule was sealed immediately and heated at 175°C for 1 hr. After cooling, the ampule was broken and the formic acid was removed by rotary evaporation. The dried hydrolysate was stored at -20°C.

The formic acid hydrolysis is preferred over the perchloric acid hydrolysis because of the greater ease in processing several samples simultaneously.

#### Chromatographic Separation of Pyrimidines

The HPLC conditions were as follows:

- column: analytical 25 cm, strong cation exchange, Whatman Partisil-10
- mobile phase: $NH_4H_2PO_4$, pH 2.0, 2 ml/min, 60 to 100 mM

- inject the equivalent of 500 μg DNA and elute with water for 10 min, then with buffer. Thymine elutes with water at 1.9 min; residual guanine elutes soon after the changeover to buffer, at 14.1 min; cytosine elutes at 14.7 min, residual adenine at 15.6 min, and 5-methylcytosine at 17.0 min. A buffer of weaker ionic strength can improve the resolution, depending on the characteristics of the individual columns.

**Methylation of Liver DNA After Administration of Various Doses of Hydrazine to Hamsters**

The dose-response study on liver DNA methylation in hamsters treated with hydrazine reported earlier (Shank et al., 1982) was repeated with more doses and a larger range than previously used. The hamster appears to be resistant to hydrazine, compared to the rat and mouse, and can tolerate more than 90 mg hydrazine/kg body weight.

Young male Syrian golden hamsters, obtained from Charles River Breeding Laboratories (Wilmington, MA), were given 0, 15, 30, 45, 60, 75, 90, 105, 120 mg hydrazine/kg body weight in 0.1 M HCl by stomach tube and decapitated 24 hr later; six animals were used per dose, and liver DNA was isolated from pooled livers from two animals per isolation. The results of the chromatographic analyses of the DNA are given in Table 1. These levels of alkylation are greater than observed in earlier experiments; these levels of 7-methylguanine closely approximate those obtained in the rat with the same doses of hydrazine (Shank et al., 1982). The levels of $O^6$-methylguanine are considerably higher in hamster liver DNA compared to the rat 24 hours after hydrazine administration; this greater concentration of $O^6$-methylguanine in hamster liver DNA probably reflects the slower rate of $O^6$-methylguanine repair reported in hamster (Stumpf et al., 1979). The amount of $O^6$-methylguanine relative to 7-methylguanine in liver DNA 24 hours after administration of hydrazine to hamsters does not appear related to dose, and thus it is unlikely that the increased persistence of $O^6$-methylguanine in hamster liver DNA is due to inhibition of $O^6$-methylguanine removal by hydrazine itself.

**Time-Response for DNA Methylation in Hamsters Treated with Hydrazine**

A study on the variation with time in the levels of 7-methylguanine and $O^6$-methylguanine in liver DNA of hamsters given 90 mg hydrazine/kg body wt was reported earlier (Shank et al., 1982). In that study 96 hr was the longest time after hydrazine administration at which analyses were done. The study was repeated here and the observation time was extended to 120 hr after hydrazine administration; also, determination of the variation with time in the levels of 5-methylcytosine in liver DNA in both controls and hamsters treated with 90 mg hydrazine/kg body wt was included in the protocol.

Young male Syrian golden hamsters were given orally 90 mg hydrazine/kg body wt and killed 0.50, 1, 3, 6, 12, 24, 48, 72, 96, and 120 hr later; controls were killed at the same intervals except for 48 and 96 hr where no control animals were included. Liver DNA has been analyzed for 7-methylguanine, and $O^6$-methylguanine; analyses for 5-methylcytosine were delayed.

As in the earlier experiments, the $O^6$-methylguanine in liver DNA from hamsters treated with hydrazine persisted for the duration of the study, up to 120 hr after toxicant administration. The results are presented graphically in Figure 1 which compares the present data to those obtained in earlier experiments in the rat and hamster (Shank et al., 1982). These results suggest that chronic administration of hydrazine to hamsters should result in the accumulation of $O^6$-methylguanine in liver DNA, and, if given sufficient time, the animals should develop liver cancer.

Table 1

Methylguanines in Liver DNA 24 Hours After Administration of Various Doses of Hydrazine to Hamsters

| Dose (mg/kg BW) | Amount of Methylguanine (μ mol/mol guanine) | | |
|---|---|---|---|
| | 7-Methylguanine | $O^6$-Methylguanine | Ratio, $O^6$:7 |
| 15 | 147 | 14 | 0.10 |
| | 181 | 12 | 0.07 |
| | 167 | 19 | 0.11 |
| | mean = 165 | mean = 15 | mean = 0.09 |
| 30 | 191 | 55 | 0.29 |
| | 209 | 20 | 0.10 |
| | 217 | 22 | 0.10 |
| | mean = 206 | mean = 32 | mean = 0.16 |
| 45 | 586 | 80 | 0.14 |
| | 391 | 53 | 0.14 |
| | 343 | 31 | 0.09 |
| | mean = 440 | mean = 55 | mean = 0.12 |
| 60 | 412 | 54 | 0.13 |
| | 544 | 78 | 0.14 |
| | 380 | 60 | 0.16 |
| | mean = 446 | mean = 64 | mean = 0.14 |
| 75 | 650 | 107 | 0.16 |
| | 572 | 96 | 0.17 |
| | 531 | 93 | 0.18 |
| | mean = 584 | mean = 98 | mean = 0.17 |
| 90 | 835 | 133 | 0.16 |
| | 820 | 149 | 0.18 |
| | 774 | 145 | 0.19 |
| | mean = 810 | mean = 142 | mean = 0.18 |
| 105 | 1077 | 175 | 0.16 |
| | 621 | 86 | 0.14 |
| | 876 | 130 | 0.15 |
| | mean = 858 | mean = 130 | mean = 0.15 |
| 120 | 1060 | 164 | 0.15 |
| | 888 | 152 | 0.17 |
| | 1473 | 193 | 0.13 |
| | mean = 1140 | mean = 170 | mean = 0.15 |

**Liver DNA Methylation in Rats Treated with Hydrazine in Different Chemical Forms**

In a proposed experiment designed to investigate the chronic toxicity of hydrazine in hamsters, the toxicant is to be administered as the sulfate in the drinking water to permit comparison of results with those reported in the literature; the animals are to receive approximately 6, 11, and 17 mg toxicant/kg body wt as hydrazine. These doses of hydrazine had little effect on the gross appearance of livers taken from animals over nine weeks of exposure (results of an experiment reported below); in contrast, rats given 4 daily administrations of 3 mg hydrazine (in 0.1 M HCl as vehicle)/kg body wt by gavage tolerated

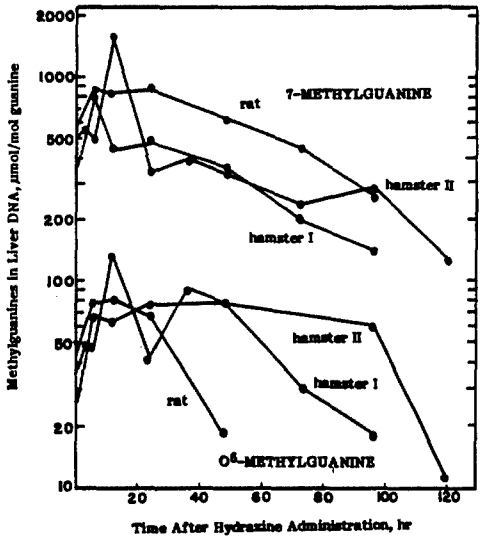


Figure 1. Methylguanines in liver DNA after administration of a single oral dose of 90 mg hydrazine/kg body weight to rats and hamsters (Hamster II indicates data from present experiment).

the exposure poorly and developed a fatty liver by the third day (Becker et al., 1981). For the 3 mg dose, hydrazine was prepared by adding 0.89 ml (28.1 mmol) hydrazine to 9.11 ml 0.1 M HCl (0.911 mmol) so that the molar ratio of hydrazine to hydrochloric acid was approximately 30:1 (the pH of the hydrazine solution was 9.85). Thus, the hydrazine was administered essentially as the free base. A small experiment was carried out to determine whether the chemical form in which inorganic hydrazine is administered makes any difference to the DNA methylation response and, thus, would influence the design of the chronic experiment.

Two young male Fischer 344 rats (Charles River Breeding Labs., Wilmington, Mass.) per group were treated as indicated in Table 2. The animals were decapitated 24 hr after treatment, and DNA was isolated and purified from individual livers. The amounts of 7-methylguanine and $O^6$-methylguanine were determined and the results are summarized in Table 3. There was little difference between the methylation levels of the DNA from the various animals, and therefore, the chemical form in which hydrazine was used to make up the dosing solutions appeared to make no difference in the methylation of liver DNA. The methylation levels were about half of what they usually are for a dose of 90 mg hydrazine/kg body wt (0.281 mmol/100 g BW, the molar dose used in this experiment), and perhaps this could be explained by the more dilute preparations used in the current experiment (administration volume of 0.75 ml compared to the usual 0.1 ml). The results of this experiment indicate that the design of the chronic experiment in which hydrazine is to be

Table 2

Protocol for Study on Various Hydrazine Solutions

| Form[a] | Molarity[b] | Vehicle | Admin. Vol.[c] |
|---|---|---|---|
| Hydrazine | 375 mM | 0.1 M HCl | 0.75 ml/100 BW |
| Hydrazine • $H_2O$ | 375 mM | water | 0.75 ml/100 BW |
| Hydrazine • $H_2SO_4$ | 375 mM | water | 0.75 ml/100 BW |
| Hydrazine • $H_2SO_4$ | 375 mM | pH 7[d] | 0.75 ml/100 BW |
| Hydrazine • 2HCl | 375 mM | water | 0.75 ml/100 BW |
| Hydrazine • 2HCl | 375 mM | pH 7 | 0.75 ml/100 BW |

[a] hydrazine (free base) and hydrazine sulfate obtained from MCB; hydrazine hydrate obtained from Aldrich; hydrazine dihydrochloride obtained from Mallinckrodt.

[b] concentration of hydrazine as administered

[c] volume of solution given by stomach tube

[d] hydrazine complex was mixed with water and pH of solution was adjusted to 7.0 with NaOH

Table 3

Liver DNA Methylation with Various Hydrazine Solutions

| Form | Liver DNA Methylation (μmol methylguanine/mol guanine) | |
|---|---|---|
| | 7-Methylguanine | $O^6$-Methylguanine |
| Hydrazine in O.1 M HCl | 362 | 25 |
| | 319 | 20 |
| | mean = 341 | mean = 23 |
| Hydrazine•$H_2O$ in water | 266 | 17 |
| | 299 | 17 |
| | mean = 283 | mean = 17 |
| Hydrazine•$H_2SO_4$ in water | 363 | 33 |
| | 300 | 20 |
| | mean = 332 | mean = 27 |
| Hydrazine•$H_2SO_4$ pH 7 | 330 | 25 |
| | 250 | 6 |
| | mean = 290 | mean = 16 |
| Hydrazine•2HCl in water | 375 | 21 |
| | 475 | 55 |
| | mean = 425 | mean = 38 |
| Hydrazine•2HCl pH 7 | 209 | 8 |
| | 295 | 17 |
| | mean = 252 | mean = 13 |

administered in the drinking water as hydrazine sulfate should be comparable to the acute studies in which hydrazine has been administered as the free base in a dilute HCl vehicle.

**Liver DNA Methylation in Hamsters During Subchronic Treatment with Hydrazine**

The time-response studies showed that, in hamsters given a single administration of hydrazine, $O^6$-methylguanine persists in liver DNA much longer than it does in rat liver DNA; this long persistence of the promutagenic lesion should greatly increase the carcinogenicity of hydrazine in the hamster, if the liver could be repeatedly exposed to levels of hydrazine to force the accumulation of this base in DNA. A chronic study in the hamster is being designed to determine whether hydrazine could be a liver carcinogen under such a regimen.

A subchronic test was done to determine the dose of hydrazine to be used in the chronic toxicity study (Bosan and Shank, 1983); young male Golden Syrian hamsters (average body weight of 59 g) were purchased from Charles River Breeding Laboratories, held in the laboratory for one week and then fasted overnight. Hydrazine was administered as hydrazine sulfate ($H_2N$-$NH_2 \cdot H_2SO_4$) at concentrations in the drinking water of 0.017-0.026%, 0.034-0.053%, and 0.051-0.083%, to provide doses of 23, 46, and 69 mg hydrazine sulfate per kg body wt. (6, 11, and 17 mg hydrazine/kg body wt.), assuming that each hamster drank an average of 8 ml water per day. An attempt was made to keep these doses constant throughout the experiment by adjusting the concentrations of the hydrazine sulfate solutions for changes in body weights and water consumption. Animals were weighed, water consumption was measured, and hydrazine sulfate solutions were prepared fresh daily for the first 10 days, and then every other day thereafter. The water bottles were wrapped in aluminum foil to protect the hydrazine from light. Groups of four control hamsters were killed at zero time and on the 6th and 63rd days of the experiment; groups of four treated animals at each dose were killed on the 2nd, 4th, 6th, 8th, 10th, 28th, and 63rd days of the experiment.

The animals were weighed (as groups) daily for the first two weeks of the drinking water exposure; during this time, little effect of the hydrazine sulfate could be detected on the growth rates of the hamsters until the 12th day of the experiment, when it became apparent that the treated animals were gaining weight at a reduced rate compared to controls. Figure 2 shows the growth rates for the control and three treated groups of hamsters over the duration of the experiment. The depression of growth rate was dose-dependent; growth rates were 90, 85, and 80% of control for the low, middle, and high doses, resp.

DNA was prepared from pools of two livers per group (three pools per group) at each time point and analyzed for 7-methylguanine and $O^6$-methylguanine (5-methylcytosine analyses were delayed); the results are shown in Figures 3 and 4. The remarkable accumulation of the promutagenic base, $O^6$-methylguanine (Fig. 4), was consistent with the slow removal of this base observed in the single exposure study and indicates that the three doses used in this experiment are appropriate for use in the planned two-year study to test for hepatocarcinogenicity.

**Various Hepatotoxins and Liver DNA Methylation**

Methylation of liver DNA following the administration of the hepatotoxins, aflatoxin $B_1$, phosphorus, diethylnitrosamine and N-nitrosopyrrolidine was measured in this series of experiments.

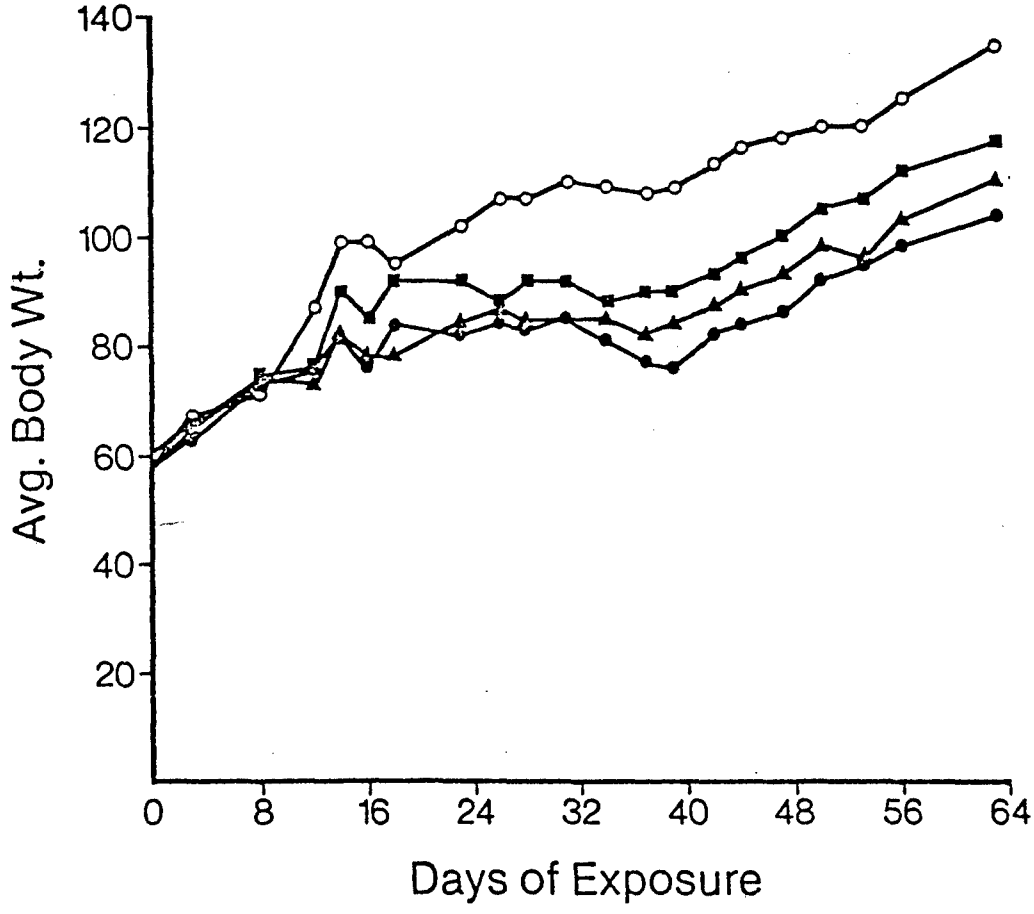


Figure 2. Average body weight (g) of hamsters given hydrazine sulfate in the drinking water at three concentrations for 9 weeks. Doses: control (open circles); 23 mg/kg body wt (squares); 46 mg/kg body wt (triangles); 69 mg/kg body wt (closed circles)

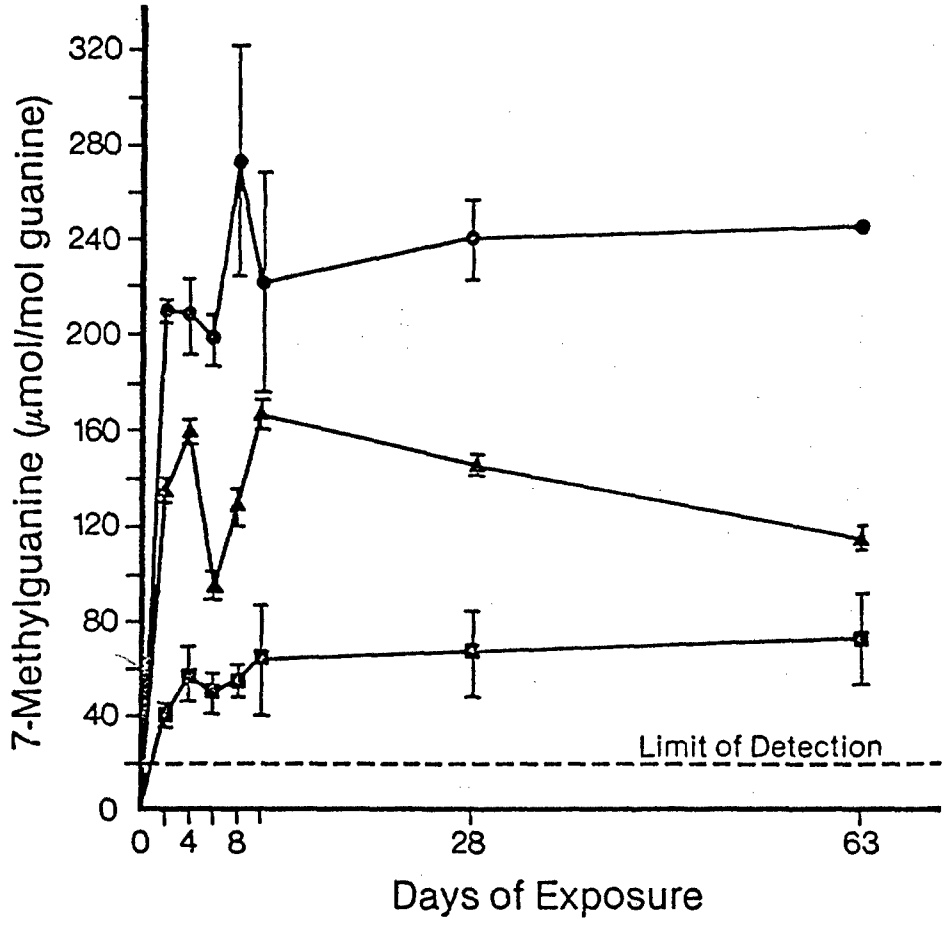


Figure 3. Levels of 7-methylguanine in liver DNA of hamsters given hydrazine sulfate in the drinking water at three concentrations for 9 weeks. Doses: 23 mg/kg body wt (squares); 46 mg/kg body wt (triangles); 69 mg/kg body wt (closed circles); control - no 7-methylguanine was detected. Bars represent range of individual values.

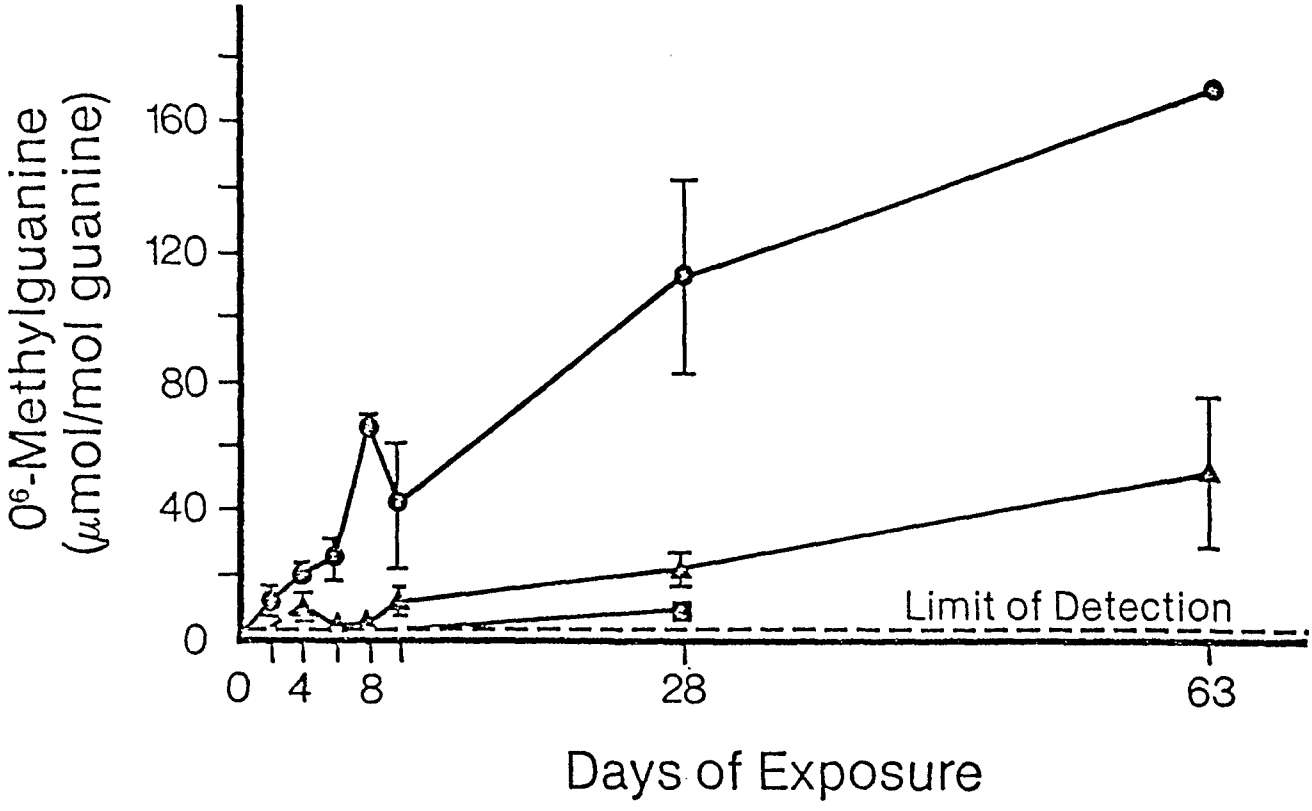


Figure 4. Levels of $O^6$-methylguanine in liver DNA of hamsters given hydrazine sulfate in the drinking water at three concentrations for 9 weeks. Doses: 23 mg/kg body wt (squares); 46 mg/kg body wt (triangles); 69 mg/kg body wt (closed circles); control - no $O^6$-methylguanine was detected. Bars represent range of individual values.

Aflatoxin and Phosphorus

Young male Golden Syrian hamsters and Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, held in the laboratory for one week, fasted overnight and then given aflatoxin $B_1$ ip in dimethylsulfoxide, or yellow phosphorus po in olive oil; 5 rats and 5 hamsters received 2 mg aflatoxin $B_1$/kg body wt, 5 rats and 6 hamsters received 6 mg aflatoxin $B_1$/kg body wt, 5 rats and 5 hamsters received 2 mg phosphorus per kg body wt.,

6 rats and 5 hamsters received 8 mg phosphorus/kg body wt., and 5 rats and 5 hamsters received 0.3 ml dimethylsulfoxide/50 g body wt ip (controls).

The rats given 8 mg phosphorus appeared ill within 4 hr; 4 out of the 6 treated rats died within 24 hr; all hamsters survived the phosphorus treatment. The rats receiving the high dose of aflatoxin $B_1$ appeared ill within 4 hr but survived the 24 hr period of the experiment.

Twenty-four hr after treatment, surviving rats and hamsters were decapitated and liver DNA was isolated and purified. The DNA specimens were analyzed for 7-methylguanine and $O^6$-methylguanine by high performance liquid chromatography, but none could be detected.

The effect of phosphorus concentration in the oil was tested, because in the earlier experiments in which 7-methylguanine and $O^6$-methylguanine were detected in liver DNA of rats given phosphorus, the toxicant was administered in a 0.5% solution in olive oil; in the above experiment, in which these bases could not be detected, the concentration of phosphorus in olive oil was 0.1%. Also, in an earlier experiment on DNA methylation after administration of hydrazine in different chemical forms, injection of hydrazine in a volume of 0.1 ml vehicle produced twice the methylation as the same dose injected in a volume of 0.75 ml.

Young male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA), held in the vivarium for a week, and fasted overnight before the experiment. Ten rats were given 0.5% phosphorus in olive oil (0.1 ml/100 g body wt, ip) and ten were given oil only (controls). Six animals were given 0.25% or 0.09% phosphorus in oil (0.2 or 0.55 ml/100 g body wt). Four animals in the 0.5% phosphorus group died within 24 hr as did 3 animals in the 0.09% group; no animals in the 0.25% group died. All surviving animals were killed 24 hr after treatment and the livers were frozen for subsequent DNA isolation. Livers from phosphorus-treated animals were slightly pale, and this effect was most prominent in the animals treated with the 0.25% phosphorus solution. The DNA samples were analyzed for the presence of 7-methylguanine and $O^6$-methylguanine by fluorescence high pressure liquid chromatography using the new, highly sensitive Perkin Elmer system; neither methylguanine could be detected in any of the samples.

Diethylnitrosamine

Diethylnitrosamine (DEN) is a hepatotoxin and hepatocarcinogen and known to be metabolized to a DNA-ethylating agent (Magee and Lee, 1964; Swann and Magee, 1971); because it is a hepatotoxin, diethylnitrosamine was tested to determine whether its administration to animals could result in S-adenosylmethionine-dependent methylation of DNA guanine.

Two Fischer 344 rats (1665 and 185g) were each given ip 216 mg DEN/kg body wt and immediately thereafter administered 100 μCi (methyl-$^3$H)methionine (specific activity, 15 Ci/mmol) each, by ip injection. (Methyl-$^3$H)methionine injections were repeated hourly; each rat was given a total of 1.2 mCi and was killed 13 hr after DEN treatment. Liver DNA was isolated from pooled livers, and subjected to mild acid hydrolysis. 7-Methylguanine, 7-ethylguanine, $O^6$-methylguanine and $O^6$-ethylguanine were added as chromatographic markers, and the hydrolysate was fractionated by liquid chromatography. Fractions corresponding to pyrimidine oligonucleotides, guanine, adenine, 7-methylguanine, 7-ethylguanine, $O^6$-methylguanine, and $O^6$-ethylguanine were collected. The pyrimidine oligonucleotide fraction was evaporated to dryness, hydrolyzed in perchloric acid, and fractionated by liquid chromatography, and the thymine, cytosine and 5-methylcytosine

fractions were collected. All fractions were concentrated and/or normalized to equivalent salt concentration by ion exchange liquid chromatography and assayed for radioactivity by liquid scintillation spectroscopy.

In a second attempt to measure aberrant methylation of liver DNA in rats treated with DEN, two rats (255 and 265 g body wt) were treated with 216 mg DEN/kg body wt ip. In contrast to the first experiment with DEN, administration of tritiated methionine was delayed; one rat was given 100 μ Ci/hr for 5 hr beginning 12 hr after DEN treatment, and the second rat received the same amount beginning 24 hr after treatment with DEN. In both cases the animals were killed 1 hr after receiving the last dose of methionine, correspondidng to 17 and 29 hr after DEN treatment. The results of these two experiments are summarized in Table 4. Radioactivity coeluting with carrier 7-methylguanine was detected in the DNA hydrolysate prepared from the rat given labeled methionine 12-16 hr after DEN treatment; however, more radioactivity eluted in the adenine fraction immediately preceding the elution of 7-methylguanine, preventing unequivocal interpretation of the data. Incorporation of radioactivity into liver DNA fraction from the rat given labeled methionine 24-28 hr after DEN treatment was also equivocal. Significant amounts of radioactivity were detected in both $O^6$-methylguanine and 7-ethylguanine fractions, while only background radioactivity was found in the 7-methylguanine fraction. The source of this radioactivity is unknown; however, it is unlikely that this radioactivity represents transfer of methyl moieties to the N-7 and $O^6$ atoms of guanine. The incorporation of detectable amounts of tritium in the $O^6$-methylguanine fraction is inconsistent with the failure to detect significant radioactivity in the 7-methylguanine fractions concomittantly. In addition, it is unlikely that treatment with tritiated methionine would lead to the formation of radiolabeled 7-ethylguanine.

Table 4

Incorporation of Tritium into Liver DNA Fractions from DEN-Treated Rats Given ($^3$H-Methyl)Methionine

| DNA Fraction | Tritium Incorporation (dpm/mg DNA) Methionine administration followed DEN treatment by 0 hr[a] | 12 hr[b] | 24 hr[b] |
|---|---|---|---|
| Thymine | 80 | -[c] | - |
| Cytosine | 111 | - | - |
| 5-Methylcytosine | 92 | - | - |
| Pyrimidine Oligonucleotides | - | 103 | - |
| Guanine | 1 | 4 | 9 |
| Adenine | 15 | 11 | 28 |
| 7-Methylguanine | ND[d] | 4 | ND |
| $O^6$-Methylguanine | ND | ND | 65 |
| 7-Ethylguanine | ND | ND | 5 |
| $O^6$-Ethylguanine | ND | ND | ND |

[a] ($^3$H-methyl)methionine administered in 12 hourly injections.
[b] ($^3$H-methyl)methionine administered in 5 hourly injections.
[c] -, not determined.
[d] ND, none detected < 0.05 dpm/mg DNA).

With the failure of these radiolabel experiments to support or refute the formation of methylguanines in DNA of DEN-treated animals, a small experiment was done in which

detection of the methylguanines was attempted using the sensitive fluorescence liquid chromatography method.

One Sprague Dawley rat (173 g) was given 200 mg DEN/kg body wt and killed 24 hr later; 3 Sprague Dawley rats were treated daily with 100 mg DEN/kg body wt for 5 days and killed 25 hr after the last dose. Liver DNA was subjected to neutral thermal hydrolysis to preferentially release 7-alkylguanine moieties, and the hydrolysates were analyzed by liquid chromatography; 7-ethylguanine, but not 7-methylguanine, was detected, leading to the conclusion that administration of toxic doses of DEN does not appear to result in the formation of methylguanines.

N-Nitrosopyrrolidine

N-Nitrosopyrrolidine is also a hepatotoxin and hepatocarcinogen, and it, too, was tested in the aberrant DNA methylation assay. Twelve rats (150-200 g) were fasted overnight, given 50 or 900 mg N-nitrosopyrrolidine/kg body wt in saline by stomach tube and killed 24, 48, 72, 96, 120, or 144 hr later. Liquid chromatographic analysis of the liver DNA detected trace amounts of μmol 7-methylguanine in 4 of the 24 treated animals (Table 5). No 7-methylguanine could be detected in liver DNA prepared from three control animals killed at 24, 48, 72, or 96 hr. DNA from kidney and lung from both treated and control rats was also analyzed for 7-methylguanine and none was detected.

Table 5
Formation of 7-Methylguanine in Liver DNA of Rats Treated with N-Nitrosopyrrolidine (NP)

| Dose (NP) (mg/kg body wt) | Kill Time (hr) | 7-Methylguanine (μmol/mol guanine) |
|---|---|---|
| 50 | 72 | 15 |
| 900 | 24 | 50 |
| 900 | 96 | 97 |
| 900 | 144 | 45 |

A similar experiment was performed with hamsters, which appear to be more sensitive to the acute toxicity of nitrosopyrrolidine. Fourteen hamsters (65-85 g) were fasted overnight and given by gavage 225 mg nitrosopyrrolidine/ kg body wt in saline; three controls were given saline only. Nitrosopyrrolidine-treated animals were killed 12, 24, 36, 48, 72, 96, or 120 hr after treatment; two controls were killed 24 hr after receiving saline, and the remaining control animal was killed 96 hr after saline administration. No 7-methylguanine was found in kidney or lung DNA from any of the animals or in liver DNA from the control animals. The amounts of 7-methylguanine in liver DNA from nitrosopyrrolidine-treated animals were barely detectable and not related to the time from treatment to death (Table 6).

Although 7-methylguanine in liver DNA could be demonstrated in rats and hamsters given nitrosopyrrolidine, the amount of 7-methylguanine did not appear to be related to either dose or time after administration. The levels of 7-methylguanine were near the limit of detection, and this may explain the inability to demonstrate dose and time dependency. Analysis of comparable amounts of control DNA and DNA from non-hepatic tissue did not contain detectable levels of 7-methylguanine, making unlikely the explanation that the 7-methylguanine in liver DNA of nitrosopyrrolidine-treated animals represents contamination of that DNA by RNA (in which 7-methylguanine is a normal constituent).

Table 6

Formation of 7-Methylguanine in Liver DNA of Hamsters Treated with N-Nitrosopyrrolidine

| Kill Time (hr) | 7-Methylguanine (μ mol/mol guanine) |
|---|---|
| 12 | 25 |
| 12 | 18 |
| 24 | 19 |
| 24 | 31 |
| 36 | 21 |
| 36 | 37 |
| 48 | 18 |
| 48 | 20 |
| 72 | 111 |
| 72 | 26 |
| 96 | 26 |
| 96 | 32 |
| 120 | ND (< 5) |
| 120 | 21 |

**Effect of Ethanol on DNA Methylation in Rat Brain**

A series of investigations was begun to determine whether the DNA methylation response observed in livers of rats treated with hydrazine occurs in other tissues of animals treated with tissue-specific toxicants. The first such system tested was the effect of ethanol on DNA methylation in brain.

Six young male Sprague Dawley rats were given 5 g ethanol/kg body weight by intubation; the dose was divided into thirds and administered as 70% ethanol in water every 30 minutes. Control rats were given equal volumes of water only. All rats were killed 24 hr after treatment and DNA was isolated from brain and liver. Chromatographic analysis failed to detect any methylguanines in the DNA of brain or liver.

**Direct Methylation of DNA Guanine by S-Adenosylmethionine**

Barrows and Magee (1982), Rydberg and Lindahl (1982), and Naslund et al., (1983) reported evidence for the direct non-enzymatic methylation of DNA purines (7-methylguanine, $O^6$-methylguanine, and 3-methyladenine) by S-adenosylmethionine, which supported an earlier hypothesis from our laboratory that hydrazine toxicity may induce the direct, non-enzymatic methylation of liver DNA by S-adenosylmethionine in the poisoned animal (Becker et al., 1981).

In a collaborative study with Dr. Louis R. Barrows of the Fels Research Institute in Philadelphia, naked calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was incubated in 0, 0.2, 1.0, or 5.0 mM S-adenosylmethionine in phosphate buffer, pH 7.4. The DNA was isolated, purified and subjected to neutral thermal hydrolysis to preferentially release 7-methylguanine. The amount of 7-methylguanine in the DNA, measured by fluorescence liquid chromatography, increased with increasing concentrations of S-adenosylmethionine in the incubation system: control and 0.2 mM S-adenosylmethionine, no detectable 7-methylguanine; 1 and 5 mM S-adenosylmethionine, 47 and 102 μ mol 7-methylguanine/mol guanine, resp. Chromatographic analysis of mild acid hydrolysates of the DNA recovered from these incubations (a few milligrams of DNA each) failed to detect any $O^6$-methylguanine or 3-methyladenine (which fluoresces weakly).

**Effect of Methionine and Hydrazine on DNA Methylation in Liver of Guinea Pigs**

It has been reported that the guinea pig is not able to regulate the level of S-adenosylmethionine in the liver and that administration of methionine to the guinea pig causes the hepatic pool of S-adenosylmethionine to increase sharply (Hardwick et al., , 1970). Since it has been shown that S-adenosylmethionine can directly methylate DNA in in vitro studies (Barrows and Magee, 1982), it was decided to use the guinea pig model to determine whether increasing the S-adenosylmethionine pool in the liver results in the formation of 7-methylguanine and $O^6$-methylguanine in liver DNA.

Adult female Hartley guinea pigs (435-720 g, a gift of the City of Hope National Medical Research Center, Duarte, California) were given 6.6 mmol methionine/kg body weight and, 4 hr later, 90 mg hydrazine/kg body weight. At this dose of hydrazine the animals were moribund one hour after treatment and were killed at that time. Hepatic levels of S-adenosylmethionine were determined quantitatively from purified acid extracts of liver homogenates and fractionation by high pressure liquid chromatography on a strong cation exchange column with detection at 254 nm as described earlier (Shank et al., 1982); liver DNA was analyzed for the presence of 7-methylguanine and $O^6$-methylguanine. The results are summarized in Table 7.

Stimulation of S-adenosylmethionine production in the guinea pig in this experiment (control, 34 nmol S-adenosylmethionine/g liver; methionine-treated, 672 nmol S-adenosylmethionine/g liver) was even greater than reported by Hardwick and coworkers in 1970 (control, 36 nmol S-adenosylmethionine/g liver; methionine-treated, 266 nmol S-adenosylmethionine/g liver). The administration of hydrazine appeared to reduce the level of S-adenosylmethionine in the guinea pig liver appreciably. In spite of the large increase in the hepatic S-adenosylmethionine pool following methionine administration, there was no detectable methylation of liver DNA, and little or no influence on the methylation of liver DNA in hydrazine-treated animals. This would suggest, then, that S-adenosylmethionine itself, in this in vivo system, may not directly methylate DNA in normal or hydrazine-treated animals. The amount of DNA methylation in hydrazine-treated animals appears slightly lower in those guinea pigs in which the S-adenosylmethionine pool had been greatly expanded, although the apparent differences in methylation levels may be within experimental error.

**Effect of the Hepatic 1-Carbon Pool on DNA Methylation in Hydrazine Toxicity**

In a review article written by Biancifiori and Severi and published in 1966, reference was made to a personal communication from F. L. Rose, who suggested that hydrazine might react with endogenous formaldehyde or a functional derivative, such as hydroxymethylfolic acid, to form a hydrazone which could then be oxidized to the strong methylating agent, diazomethane:

Table 7

S-Adenosylmethionine and DNA Methylguanine Levels in Liver after Administration of Methionine and Hydrazine to Guinea Pigs

| Group | AdoMet[a] (nmol/g liver) | | Methylguanines (μ mol/mol DNA guanine) 7-methylguanine | | $O^6$-methylguanine | |
|---|---|---|---|---|---|---|
| Control | 44 | | ND [b] | | ND | |
| | 24 | | ND | | ND | |
| | | mean = 34 | | | | |
| Hydrazine | 14 | | 396 | | 27 | |
| | 15 | | 328 | | 25 | |
| | | mean = 15 | | mean = 362 | | mean = 26 |
| Methionine | 706 | | ND | | ND | |
| | 638 | | ND | | ND | |
| | | mean = 672 | | | | |
| Methionine-Hydrazine | 476 | | 280 | | 18 | |
| | 441 | | 160 | | 13 | |
| | | mean = 458 | | mean = 220 | | mean = 16 |

[a] AdoMet = S-adenosylmethionine
[b] ND = none detected

$$HCHO + H_2N\text{-}NH_2 \longrightarrow \left[HC(OH)NH\text{-}NH_2\right] \longrightarrow CH_2{=}N\text{-}NH_2 \longrightarrow {}^{-}CH_2\text{-}{}^{+}N{\equiv}N$$

The fact that the methyl source in the hydrazine/DNA methylation response can be labeled by either $^{14}$C-formate or ($^{14}$C-methyl)methionine (Barrows and Shank, 1978; Quinter-Ruiz et al., 1981) is consistent with this hypothesis.

Three rats were used in a preliminary experiment to test this hypothesis. Three rats (177-189 g) were given 800 mg sodium formate/kg body wt intraperitoneally to greatly expand the 1-carbon pool in the liver; 15 min later each rat was given 90 mg hydrazine/kg body wt by stomach tube, and the animals were decapitated 6 hr later. Pretreatment of the animals with formate should have provided a relatively large pool of formaldehyde (relative to hepatocytes in untreated animals) with which the hydrazine could react to form diazomethane, as proposed above. It is already known (Becker et al., 1981) that administration of 90 mg hydrazine/kg body wt to the rat results in levels of about 800 micromoles 7-methylguanine and 80 micromoles $O^6$-methylguanine per mole guanine in 6 hr; thus, in the liver DNA of these animals, considerably (possibly an order of magnitude) greater methylation of DNA guanine should be found, however, the DNA contained no detectable methylguanines.

A second attempt to alter the level of the hepatic 1-carbon pool was based on the following rationale. Methanol was administered to provide a source for the metabolic formation of formaldehyde; disulfiram was given to inhibit aldehyde dehydrogenase and thus prolong the persistence of formaldehyde in the liver; formate was administered in another attempt to increase the level of the 1-carbon pool in the liver. Semicarbazide was given to

compete with hydrazine for the available formaldehyde and possibly 'protect' against DNA methylation.

Young adult Sprague-Dawley rats (218-275 g) were fasted overnight and then treated as follows: at T = -4 hr rats were given 500 mg disulfiram/kg body weight po in corn oil (5 ml/kg BW); at T = -0.5 hr animals were treated with 1 ml methanol (100%)/kg ip, 100 mg semicarbazide HCl/kg in 1 ml water/kg po, and/or 500 mg sodium formate/kg in 2 ml water/kg ip. Hydrazine (90 mg/kg po) was given at T = 0. All animals were killed at T = 24 hr. A summary of the treatment schedule is given in Table 8 and the results are summarized in Table 9.

Table 8

Schedule for Rats Treated with Hydrazine and Modifiers of the 1-Carbon Pool

| Group | No. Rats | Hydrazine | Disulfiram | Methanol | Semicarbazide | Formate |
|---|---|---|---|---|---|---|
| 1 | 6 | - | - | - | - | - |
| 2 | 3 | + | - | - | - | - |
| 3 | 6 | - | + | - | - | - |
| 4 | 6 | - | - | + | - | - |
| 5 | 3 | + | + | - | - | - |
| 6 | 3 | + | - | + | - | - |
| 7 | 6 | - | + | + | - | - |
| 8 | 3 | + | + | + | - | - |
| 9 | 3 | - | - | - | + | - |
| 10 | 3 | + | - | - | + | - |
| 11 | 3 | + | + | + | + | - |
| 12 | 6 | - | - | - | - | + |
| 13 | 3 | + | - | - | - | + |
| 14 | 3 | - | - | - | + | + |
| 15 | 3 | + | - | - | + | + |

The combination of hydrazine and methanol proved to be fatal to all animals (Groups 6, 8, and 11); in addition, 1 out of 3 animals died after single administration of disulfiram, semicarbazide, or sodium formate alone (Groups 3, 9, and 12). The high mortality left few animals with which to complete the experiment. The few results that were obtained weakly suggest that semicarbazide, which was expected to scavenge endogenous formaldehyde, did not decrease the amount of DNA methylation (Groups 2 vs 10), and that formate, which was expected to increase the concentration of endogenous formaldehyde, did not increase the amount of DNA methylation (Groups 2 vs 13). It remains to be determined if indeed any of these treatments did alter the formaldehyde levels in the liver.

A preliminary in vitro study was also done. Liver DNA was isolated and purified from untreated Sprague Dawley rats to serve as substrate for the methylation reaction. The DNA was dissolved in 10 mM sodium cacodylate and incubated with formaldehyde and hydrazine under the following conditions: 2 mg DNA per ml buffer (based on the in vitro study on the nonenzymatic methylation of DNA by S-adenosylmethionine by Barrows and Magee in 1982), pH 7.4 (physiological), 5 mmolar hydrazine (Siemens and coworkers in 1980 showed that 2 mmolar hydrazine was toxic to cultured cells but a concentration of 0.5 mmolar was not)

Table 9

Methylguanine Levels in Liver DNA of Rats Treated with Hydrazine and Modifiers of the 1-Carbon Pool

| Group | Methylguanines (μ mol/mol guanine) 7-methylguanine | $O^6$-methylguanine |
|---|---|---|
| 1 | ND | ND |
| 2 | 660, 760, 805 | 52, 56, 100 |
| 3 | ND | ND |
| 4 | ND | ND |
| 5 | 355 | 18 |
| 6 | animals died | animals died |
| 7 | ND | ND |
| 8 | animals died | animals died |
| 9 | ND | ND |
| 10 | 260, 760 | 8, 46 |
| 11 | animals died | animals died |
| 12 | ND | ND |
| 13 | 430, 450 | 11, 14 |
| 14 | ND | ND |
| 15 | 100, 430, 730 | 21, 54, lost |

and 5 mmolar formaldehyde (equimolar to hydrazine). After a 1-hour incubation at 37°C, the DNA was precipitated with ethanol, washed, and analyzed by HPLC for 7-methylguanine and $O^6$-methylguanine. Controls consisted of DNA incubated with either hydrazine or formaldehyde alone.

The DNA incubated in either 5 mmolar hydrazine or 5 mmolar formaldehyde was considerably depolymerized (difficult to precipitate under normal conditions used for highly polymerized DNA), but the DNA incubated with the combination of 5 mmolar hydrazine and 5 mmolar formaldehyde precipated normally under the same conditions. No 7-methylguanine or $O^6$-methylguanine could be detected in any of the DNA samples using HPLC analysis.

## SUMMARY AND CONCLUSIONS

Previous carcinogenesis studies, focussing on methylation of liver DNA guanine in response to hydrazine administration have been carried out in the rat (Barrows and Shank, 1978; 1981; Becker et al., 1981). Those studies provided evidence to suggest that hydrazine may initiate carcinogenesis by causing the formation of the promutagenic base, $O^6$-methylguanine, in the liver DNA; hydrazine has been shown to induce hepatocellular carcinomas in the rat (Severi and Biancifiori, 1968). To provide further support for this hypothesis, the Syrian golden hamster was selected as an animal model. $O^6$-Methylguanine, formed after a single administration of dimethylnitrosamine, is removed from the liver of this species more slowly than it is from rat liver (Stumpf et al., 1979), which suggests that this base could accumulate in liver DNA of hamsters given repeated exposure to dimethylnitrosamine. This carcinogen does produce hepatocellular and cholangiocellular carcinomas and hemangioendotheliomas in the hamster (Tomatis et al., 1964; Mohr et al., 1974).

Repeated exposure to hydrazine sulfate in the drinking water of hamsters in the current study did indeed lead to accumulation of $O^6$-methylguanine in the liver DNA. Yet studies carried out in other laboratories have not produced liver cancer in hamsters exposed to hydrazine (Biancifiori, 1970; Toth, 1972; Dr. E. Weisburger, personal communication). The hamster exposed to hydrazine under conditions which lead to accumulation of $O^6$-methylguanine in liver DNA should provide a useful model with which to test the relevance of this methylated base to carcinogenesis.

Little work has been done on methylation of liver DNA guanine in animals treated with hepatotoxins other than hydrazine. Such a study needs a systematic approach to define the conditions necessary to obtain reproducible quantitative results with compounds such as carbon tetrachloride, ethanol, phosphorus, aflatoxin $B_1$, etc. The experiments carried out so far have involved single exposures of hepatotoxins to rats, but the results, both DNA methylation and toxicity, have been capricious. Future experiments will attempt to take advantage of the slow removal of $O^6$-methylguanine in hamster liver DNA and will use this species to examine the response to hepatotoxicity induced by a variety of agents.

More work is needed on the identification of the methylating agent in the formation of methylguanines in liver DNA of animals treated with hydrazine. S-Adenosylmethionine appears to be involved, but the evidence obtained so far does not strongly support the suggestion that hydrazine may stimulate the non-enzymatic, undirected methylation of DNA by S-adenosylmethionine . In vitro studies have confirmed that S-adenosylmethionine can non-enzymatically methylate DNA at the 7-position of guanine but not to the extent seen in hydrazine-treated animals. Also, expanding the size of the S-adenosylmethionine pool in the liver, as was done in the guinea pig, failed to change the amount of DNA methylguanines seen in response to hydrazine administration. The possibility that hydrazine may react with endogenous formaldehyde to form a methylating intermediate was recognized, but results from a preliminary experiment have not yet provided strong evidence to support this hypothesis.

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